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The Microbial Communities and Fruiting of Edible Ectomycorrhizal Mushrooms

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Abstract: The competition between edible ectomycorrhizal fungi (EMF) and other ectomycorrhizal fungi have been studied in greenhouse and in the field taking into account different environmental conditions and using molecular tools as well as morphological methods. Approximately 100 ectomycorrhizal species were identified in soil fungal communities in productive areas of *Boletus edulis sensu lato* and in truffières of *Tuber magnatum* and *Tuber borchii*. The bacterial communities occurring in *T. magnatum* and *T. borchii* fruiting bodies during different stages of their maturation were also characterized. α-Proteobacteria, were found as major constituents of the bacterial community associated with truffle ascoma, independentely of the degree of maturation. These bacteria include members of the *Sinorhizobium Ensifer* and *Rhizobium Agrobacterium* groups, and *Bradyrhizobium* spp., which are well known for their ability to fix atmospheric nitrogen.

Our studies raise two important questions about truffle biology: 1) can the composition of microbial soil communities infuence EMF fruiting body production? 2) can nitrogen fixing bacteria play a role in truffle nutrition?

Key words: Edible ectomycorrizal fungi communities; Competition; Bacteria; Truffle; Porcino
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Introduction

The edible ectomycorrhizal mushrooms (EEMM) have a market measured in billions of euros. Many, such as the European truffles *Tuber magnatum*, *Tuber borchii*, *Tuber melanosporum* and *Tuber aestivum*, and porcini (*Boletus edulis* s.l.), all of which can be found in Italy, are appreciated worldwide for their aroma and flavours (Hall *et al.*, 2007). Ecological studies on the EEMM have tended to concentrate on the soil and climatic characteristics of the production areas with generally little attention paid to the associated microbiota which a priori can be assumed to strongly interact with them (Hall *et al.*, 2003).

In the forests of the Northern Hemisphere, that are dominated by ectomycorrhizal trees, it is not unusual to find many different ectomycorrhizal fungi on the roots of a single host plant. Among these fungi there is an intense competition for space on the roots and for nutrients arising a dynamic equilibrium conditioned by the host plant, biotic factors and environmental conditions (Jumpponen and Egerton-Warburton, 2005). The soil also include numerous other microorganisms such as pathogenic and saprobic fungi, and bacteria which are well known to interact with the ectomycorrhizal fungi (Paulitz and Linderman, 1991; Cairney and Meharg, 2002; Frey-Klett *et al.*, 2007) but their relationship with EEMM was poorly investigated aforetime.

Competition between ectomycorrhizal fungi

The first studies conducted by our research group were conducted in the greenhouse, where we investigated competition between *T. borchii*, *Hebeloma sinapizans* and *Laccaria bicolor* on *Pinus pinea* seedlings (Zambonelli and Branzanti, 1990). In these experiments we showed that the competitiveness of *T. borchii*

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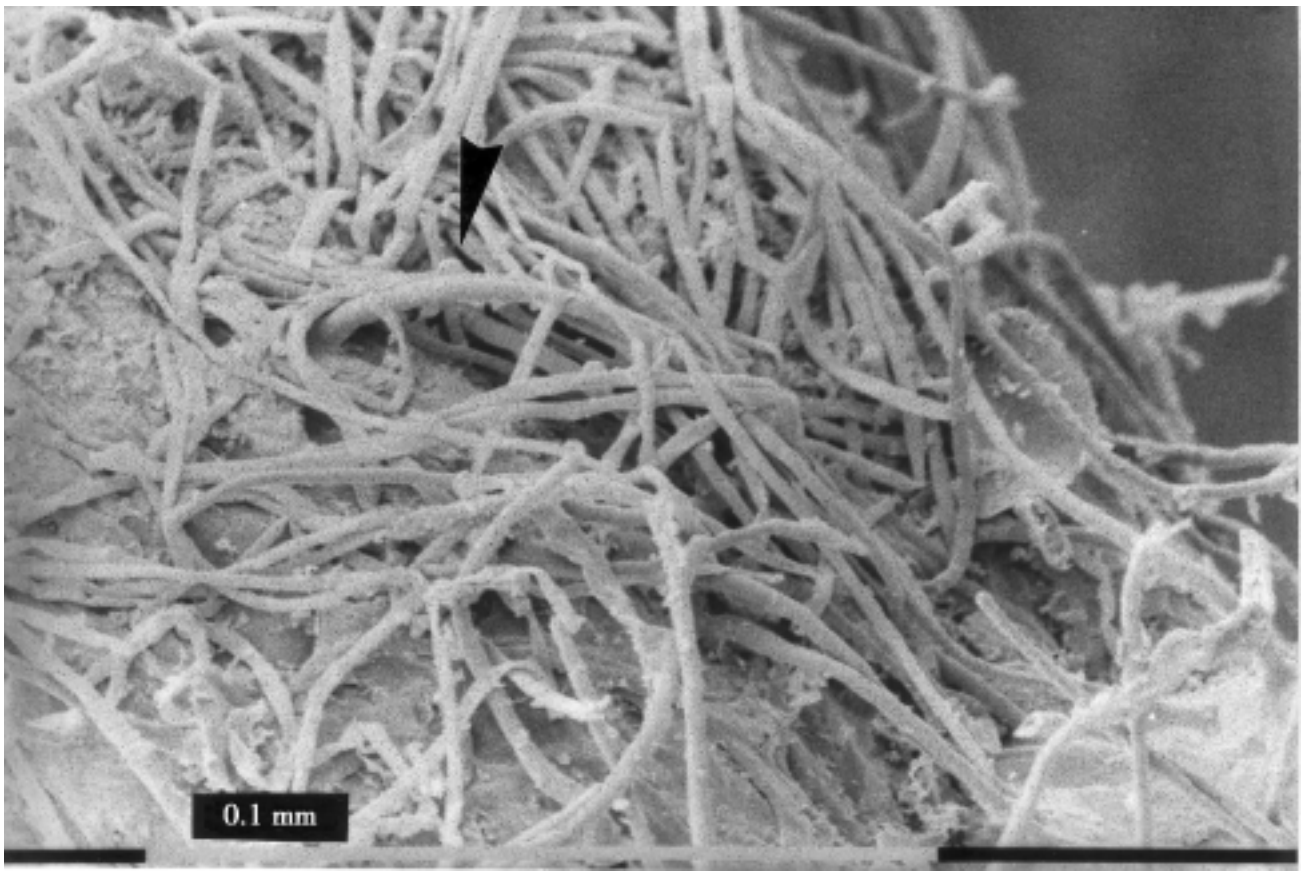

Fig . 1 Ectomycorrhiza of *Tuber borchii* invaded by *Hebeloma sinapizans*
With arrow is indicated a *T*. *borchii* cystidium bended by *Hebeloma sinapizans* (from Zambonelli *et al.*, 1995)

varied with the substrate . In peat moss and vermiculite both *H*. *sinapizans* and *Laccaria bicolor* out competed *T*. *borchii* whereas in calcareous soil only *H*. *sinapizans* was able to form abundant mycorrhizae when inoculated together with *T*. *borchii* . Similarly, in the greenhouse plants previously colonised by *T*. *borchii* were invaded and colonised by *H*. *sinapizans* after it was introduced (Zambonelli *et al.*, 1995) .

However, when the seedlings were transplanted into the field in an area particularly favourable to *T*. *borchii*, it became the most competitive fungus and mycorrhizae formed by *H*. *sinapizans*, *L*. *bicolor* and the common greenhouse contaminant *Spharosporella brunnea* completely disappeared after four years (Zambonelli *et al.*, 2000) . This experiment showed that the soil characteristics could completely change competition between EEMM and other fungi .

In a subsequent field experiment in a young *T*. *aestivum* truffière, we modified the soil conditions by mulching with different materials (Zambonelli *et al.*, 2005) . Mulching is a traditional practice in truffle cultivation where a layer of straw, leaves, branches, or plastic film is laid on the soil surface in the rooting zones of the host plants to limit the development of grasses, maintain soil moisture during drought, and increase truffle production (Fioc, 1987; Ciani *et al.*, 1988; Etayo and De Miguel, 2001) . It is well known that mulching increase soil moisture and equilibrate soil temperature but can also have long-term responses such as an increase in soil mineral nutrients, soil aggregation and drainage characteristics, microbial activity, and cellulase activity (Downer *et al.*, 2002) . As a consequence mulching can influence the development of diseases, insects, nematodes and soil borne fungi as well as plant growth and truffle yields (Elder *et al.*, 2002) .

The experimental truffière was located in a fresh and moist part of the Appennino mountains ideal for *T*. *aestivum* development . The mulching materials were wheat straw, an aluminised cloth, a black mulching cloth and a synthetic grey tissue . Three years after the start of the experiment the mulches significantly influenced the degree of ectomycorrhizal colonization in the upper 0 - 15 cm of soil and also differentially affected the competitiveness of *T*. *aestivum* against other soil fungi . Both straw and the black mulch depressed *T*. *aestivum* infection, probably because increasing the soil moisture favoured the development of contaminant ectomycorrhizal fungi . In contrast, the tissue stimulated *T*. *aestivum* colonization and depressed the development of the contaminating ectomycorrhizal fungi proba-

bly because it slightly increased soil temperatures. So the outcomes of competition are not solely affected by the competitive abilities of the component fungi in a determined soil but also other environmental factors such as temperature and water can influence competition among ectomycorrhizal fungi. These factors can be modified by mulching which can have an impact on the development of EEMM fungi.

EEMM fungal communities

In the cultivation of EEMM competition from resident ectomycorrhizal fungi is one of the biggest problems particularly in the first few years after planting when the introduced species can be replaced. In contrast, naturally the EEMM survive in a dynamic equilibrium with other ectomycorrhizal fungal species sometimes coexisting on the same root system or even on the same root tip. For example, Hall and collaborators consistently found *Boletus edulis* fruiting bodies in the same locations and at the same time of year as *Amanita muscaria* or *Amanita excelsa* in Austria, England, Italy, New Zealand, Sweden and USA (Hall *et al.*, 2003). Moreover *A. muscaria* or *A. excelsa* and *B. edulis* hyphae and rhizomorphs were often closely interwoven.

This emphasises the need to better understand the consistence and the dynamics of soil ectomycorrhizal communities.

Until recently studies on ectomycorrhizal fungal communities were only conducted using morphological and cultural techniques that were able to identify only a limited number of microbial species underestimating most of the autochthonous species that are uncultivable. The application of molecular techniques for taxonomic affiliation has dramatically changed the situation on microbial ecology, so that it is now possible to investigate the composition of microbial communities in soil, mycorrhizas and fruiting bodies and provide a more complete and precise scenario of their structural composition and dynamics (Dahlberg, 2001; Selosse, 2001).

The internal transcribed spacers (ITS region) of rDNA are the most frequently used DNA region for the identification of ectomycorrhizal fungi. The comparison between the ITS sequence obtained from those deposited in sequence data base often allows us to identify the fungal species at genus or even at the species level. It is also useful to compare the obtained ITS sequences with those of the fruiting bodies present in the studied areas using the entire ITS sequences or their RFLP profiles (Dahlberg, 2001).

In order to reduce the time and the costs of molecular analyses of mycorrhizae our research group uses a method for direct PCR of the mycorrhizal mantle so avoiding DNA extraction (Iotti *et al.*, 2006). This method is more precise than the conventional technique because examining only a small piece of fungal mantle reduces the risk of PCR contamination from other fungal species often present as mycelium on the surface of a mycorrhiza.

Ectomycorrhizal communities have been studied using molecular tools in natural *T. magnatum* truffières where it was shown that *T. magnatum* mycorrhizae are quite rare (less than 5% even in productive areas) and that the most common ectomycorrhizal fungi present are other species of *Tuber* such as *T. rufum* (Bertini *et al.*, 2006; Murat *et al.*, 2005). In contrast, a study we made of natural *T. borchii* truffières in the highly productive littoral zone near Ferrara, Italy, we found abundant *T. borchii* mycorrhizae with often more than 30% of the root tips infected by the fungus (unpublished data).

In a survey of all ectomycorrhizae, saprobic and pathogenic fungi in a *Boletus edulis sensu lato* (porcini) productive area using (Peintner *et al.*, 2007) morphological and molecular identification 39 ectomycorrhizal fungi were identified on root tips whereas 40 fungal species were found in the soil using the cloning techniques. However, the overlap between above- and below-ground fungal communities was very low with *Boletus mycelia* rare and scattered compared to other soil fungi whereas their fruiting bodies dominated the above ground fungal community. Only *B. aestivalis* ectomycorrhizas and soil mycelia were relatively abundant. In the studied areas no specific fungus-fungus association as suggested by Hall and collaborators (Hall *et al.*, 2003) was found.

These studies emphasise that some EEMM such as *Tuber magnatum* and *Boletus edulis* are able to produce large quantities of fruiting bodies supported by only rare mycorrhizas and mycelia in the soil whereas other species such as *T. borchii* are supported by a large number of infected tips . Evidently there is some unknown ecological component that is involved in fruiting body formation and development .

EEMM bacterial communities

It is now well established that some bacteria associated with mycorrhizal fungi in the soil and in the rhizosphere are able to promote the establishment of the symbiosis and very few studies were devoted on the microbe host association in EEMM productive areas . These so-called mycorrhiza helper bacteria (MHB) can be found with ecto- or endomycorrhizas and belong to various taxonomic groups but particularly fluorescent pseudomonades have been studied within these association (Frey-Klett *et al.*, 2007) .

Of particular importance has been the discovery that the bacterium *Staphylococcus pasteuri*, found on the roots of vitroplants of *Populus alba* could inhibit the growth of *T. borchii* but not *Hebeloma radicosum*, another ectomycorrhizal fungus (Barbieri *et al.*, 2005b) . Whether *S. pasteuri* commonly occurs in soil is largely irrelevant what is important is that an organism apparently unrelated to either symbiotic partner might influence the composition of the ectomycorrhizal flora in a soil . This and other interactions between EEMM and associated microrganisms, could be an explanation of specific chemiotactic behaviour and spreading of certain ectomycorrhizal fungi .

Molecular techniques present also the opportunities for visualizing how organisms interact in the soil . The use fluorescent markers that can reveal the location of the (DNA) nucleic acids of specific organisms can provide important additional information on microbial soil interactions . For example, using culture-independent and dependent 16S rRNA gene based approaches we were able to increase our taxonomic knowledge of *Tuber*-associated bacteria occurring in the *T. borchii* and *T. magnatum* fruiting bodies (Barbieri *et al.*, 2005a, c, 2007) . From this work we have been able to identify new bacterial species belonging to at least six divisions: *alpha*-proteobacteria, *beta*-proteobacteria, *gamma*-proteobacteria, Bacillaceae, Firmicutes e Bacteroidetes . Of particular interest was the constant and significant presence of *alpha*-proteobacteria, mainly represented by members of the *Sinorhizobium Ensifer* and *Rhizobium Agrobacterium* groups, and *Bradyrhizobium* spp ., well known for their ability to fix nitrogen . Moreover the use of specific oligonucleotide probes for the *alpha*-proteobacteria, have allowed to quantify the incidence of the Rhizobi-like in *T. borchii* and *T. magnatum* ascocarps, estimated to account for approximately 60% - 80% of the entire Eubacterial population present *in situ* .

This percentage appears to be independent of the degree of ascoma maturation . These bacterial species, probably underestimated in the past because of the limitations of classical cultural techniques, constitute the dominant fraction of the bacterial communities associated with the ascoma of prized white truffles yet their role has been completely ignored .

Conclusion

Althouth our knowledge of the composition of soil microbial communities has increased considerably in recent years, their influence on the EEMM biology is still poor understood . However, it is now obvious that fungi and bacteria living in association with the mycelium of EEMM, mycorrhizas and or fruiting bodies do have a direct or indirect effect on competition for space or nutrients or via molecules that may trigger morphogenetic changes .

In addition the research so far completed raise two important questions about EEMM biology: i) can the composition of microbial soil communities has infuence on the production of fruting bodies, and ii) can nitrogen fixing bacteria play a role in the nutrition of fruiting bodies in particular truffles ?

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